Cholesterol crystals activate the lectin complement pathway via ficolin-2 and MBL – implications for the progression of atherosclerosis[1,2]

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Running title: Cholesterol crystals activate the lectin complement pathway

Abstract

Cholesterol crystals (CC) play an essential role in the formation of atherosclerotic plaques. CC activate the classical and the alternative complement pathways, but the role of the lectin pathway is unknown. We hypothesized that the pattern recognition molecules (PRM) from the lectin pathway bind CC and functions as an upstream innate inflammatory signal in the pathophysiology of atherosclerosis. We investigated the binding of the PRMs mannose-binding lectin (MBL), ficolin-1, ficolin-2, and ficolin-3, the associated serine proteases, and complement activation products to CC *in vitro* using recombinant proteins, specific inhibitors as well as deficient and normal sera. In addition we examined the deposition of ficolin-2 and MBL in human carotid plaques by immunohistochemistry and fluorescence microscopy. The results showed that the lectin pathway was activated on CC by binding of ficolin-2 and MBL *in vitro*, resulting in activation and deposition of complement activation products. MBL bound to CC in a calcium dependent manner while ficolin-2 binding was calcium independent. No binding was observed for ficolin-1 or ficolin-3. MBL and ficolin-2 were present in human carotid plaques and binding of MBL to CC was confirmed *in vivo* by immunohistochemistry, showing localization of MBL around CC clefts. Moreover, we demonstrated that IgM, but not IgG bound to CC *in vitro* and that C1q binding was facilitated by IgM.

In conclusion our study demonstrates that PRMs from the lectin pathway recognize CC and provides evidence for an important role for this pathway in the inflammatory response induced by CC in the pathophysiology of atherosclerosis.

Introduction

Vascular diseases arising from atherosclerosis are among the leading causes of morbidity and death in western countries. Cholesterol crystals (CC) appear early in the development of atherosclerosis and play an essential role in the formation of atherosclerotic plaques by inducing inflammation and functioning as an endogenous danger signal (1). Cholesterol is almost water insoluble, but it is solubilized *in vivo* in lipid bilayers or micelle systems, where it serves as a stabilizing component of cell membranes and a precursor of bile salts and steroid hormones. Unbalanced cholesterol metabolism results in undesired *in vivo* precipitation of CC. Cholesterol crystallizes in a bilayer structure with an end-for-end arrangement of approximately parallel molecules (2). CC are known to activate the complement system (3-5) and induce complement dependent inflammasome activation and cytokine release in phagocytes *in vitro* (1,6,7).

Activation of the complement system is an important part of the innate immune response and is initiated when pattern recognition molecules (PRMs) recognize pathogen associated molecular patterns on foreign cells or damaged host cells. The effector functions of the complement system include elimination of invading pathogens, regulation of adaptive immunity, and maintenance of tissue homeostasis (8). The complement system is activated through three different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP).

The PRM mannose-binding lectin (MBL) is a soluble collectin, that is able to activate the LP (9,10). MBL recognizes a wide variety of microorganisms and altered self via a carbohydrate recognition domain (CRD), and distinguish between normally present self-structures and foreign structures, including microbes and damaged self, based on the orientations of hydroxyl groups on carbohydrates (11).

The LP can also be activated by ficolins (12-14). Three types of ficolins have been characterized in humans; ficolin-1 (M-ficolin), ficolin-2 (L-ficolin), and ficolin-3 (H-ficolin). The ficolins are multimeric macromolecules, structurally and functionally related to collectins. Ficolin-1, ficolin-2, and ficolin-3 are found in serum (15-17), with ficolin-1 being the least abundant (15). Ficolins primarily recognize acetylated compounds via the fibrinogen like domain, such as N-acetyl-D-glucosamine (GlcNAc) and acetylated BSA (AcBSA) (18,19). The LP recognition molecules have also been shown to recognize and participate in the removal of altered and dying host cells (20-23). PRMs of the LP are found in complexes with the MBL/ficolin-associated serine proteases (MASPs). Three catalytically active serine proteases; MASP-1, MASP-2, and MASP-3, and two alternative splice products; MAP-1 (MAp44) and sMAP (MAp19) are known (24).

The CP is activated by direct binding of the PRM C1q to various structures and ligands on pathogens or apoptotic cells, or indirectly via other molecules such as immunoglobulins or C-reactive protein. The AP is activated by spontaneous hydrolysis of the internal C3 thioester in the fluid phase or directly on foreign surfaces that are not protected against complement activation (25). In addition, the AP functions as an amplification loop, enhancing CP and/or LP initiated complement activation substantially.

Complement activation and deposition of complement activation products occur both in experimental and human atherosclerosis (26-31). Monohydrate CC, similar to those found in atherosclerotic plaques, activate the CP and the AP (3-5,7). CC activate the CP through binding of C1q, but whether C1q binds directly to the CC or indirectly via immunoglobulins, C-reactive protein, or other molecules is still not clear. C1q is present in atherosclerotic lesions and has been shown to play a protective role in early atherosclerotic development in a C1q deficient mouse

model of atherosclerosis (32). However, in this model, C5b-9 deposition in aortic lesions was not abolished suggesting involvement of the LP and/or the AP.

Whether the LP is involved in the local inflammation initiated by CC in the pathophysiology of atherosclerosis is not known, but MBL is present in atherosclerotic lesions and MBL gene variants leading to functional defects of MBL have been associated with myocardial infarction (33) and severity of atherosclerosis (34,35). Cholesterol compounds have been shown to differ in their complement activation ability, depending on the position as well as the number of hydroxyl groups (4). Since MBL and the ficolins bind hydroxyl groups, we hypothesized that MBL and ficolins bind monohydrate CC similar to those found in atherosclerotic plaques, and functions as an upstream innate inflammatory signal in the pathophysiology of atherosclerosis. Thus, the objectives of this study were to investigate the binding of the pattern recognition molecules from the lectin complement pathway (MBL, ficolin-1, -2, and -3) and the associated serine proteases (MASPs) to cholesterol crystals *in vitro* and *in vivo* in human carotid plaques, and to determine the role of the lectin pathway in the complement activation initiated by cholesterol crystals. Furthermore, the purpose was to clarify if the C1q mediated classical pathway activation on cholesterol crystals was initiated through direct binding of C1q or if C1q bound indirectly via IgG, IgM or both.

Methods

Reagents

Ultrapure cholesterol (C8667), 1-propanol (279544), RPMI 1640 medium , Erythrosine, HSA (A9731), BSA (A2153), EGTA (E3889), GlcNAc (A8625), D-Mannose (M6020), and D-(+)-Galactose (G0750) were purchased from Sigma-Aldrich. EDTA (324503) was purchased from Calbiochem. Glycergel mounting medium (C0563), Dako EnVision Plus autostainer, Polymer EnVision-HRP anti-Mouse, DAB+ Chromogen solution, Dako wash buffer (S3006), and Serum Free Protein block (X0909) was purchased from DAKO, Denmark. Purified human C4 (A108) and purified human C1q (A099) were purchased from Complement Technology, Denmark. Serum-free medium Chinese hamster ovary (CHO) CD1 was from Lonza, Denmark. Tissue-Tek O.C.T. compound was from Sakura, Japan. Haematoxylin and Saffron was from Chemi – Teknik AS, Norway. Alexa647 anti-mouse IgG Thermo Fisher Scientific, USA and Mowiol 4-88 was from Hoechst, Germany.

The following reagents were used as complement inhibitors: C5 inhibitor; eculizumab (Soliris, Alexion), C3 inhibitory peptide; compstatin Cp40 (36), C1q inhibitory mAb mouse anti-human C1q clone 85 (IgG1) (MW1828, Sanquin), MASP-1 inhibitor SGMI-1 (37), and MASP-2 inhibitor SGMI-2 (37). The following antibodies were used: FITC conjugated polyclonal rabbit anti-rat Ab (F1763, Sigma-Aldrich), FITC conjugated polyclonal goat anti-rabbit Ab (F1262, Sigma-Aldrich), FITC conjugated polyclonal goat anti-mouse Ab (F0479, DAKO), mouse IgG1κ isotype control (BD Bioscience), mouse IgG2a isotype control (BD Bioscience), rabbit IgG isotype control (Invitrogen), rat IgG1κ isotype control (BD Bioscience), mouse anti-human MBL mAb 131-10 (IgG1κ) (Bioporto Diagnostics), mouse anti-human MBL 131-01 (IgG1κ) (Bioporto Diagnostics), MBL inhibitory mAb mouse anti-human MBL 3F8 (IgG1) (38), MBL binding mAb mouse anti-

human MBL 1C10 (IgG2) (39), rat anti-human MASP-2 mAb 8B5 (HM2190, Hycult biotech), mouse anti-human complement component C5b-9 mAb (IgG2a) (011-01, Antibody Shop), polyclonal rabbit anti-human C4c Ab (Q0369, DAKO), polyclonal rabbit anti-human C1q (A0136, DAKO), rabbit anti-human IgM (0425, DAKO) and rabbit anti-human IgG (0423, DAKO). In house produced monoclonal antibodies: mouse anti-human ficolin-1 FCN106 (IgG1κ) (40), mouse anti-human ficolin-2 FCN219 (IgG2a) (16), ficolin-2 inhibitory Ab mouse anti-human ficolin-2 FCN212 (IgG1κ) (unpublished), mouse anti-human ficolin-3 FCN334 (IgG1κ) (17), and mouse anti-human MASP-1/-3/MAP-1 8B3 (IgG1κ) (41,42).

Production of recombinant proteins

Recombinant MBL, ficolin-1, -2, -3, MASP-1, -2, and -3 were produced in our laboratory as previously described (43-45). In short, the proteins were expressed in CHO-DG44 cells cultivated in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 200 nM methotrexate or by using serum-free medium Chinese hamster ovary (CHO) CD1 supplemented with 200 nM methotrexate.

Preparation of monohydrate CC

CC were prepared essentially as described by Samstad *et al.* (2014) (7). 100 mg ultrapure cholesterol was dissolved in 50 ml 1-propanol. 75 ml distilled water was added to the solution and it was left undisturbed for 15 min for the crystals to stabilize. The solution was centrifuged and the remaining 1-propanol was removed by evaporation. All steps were performed at room temperature (RT). The CC were resuspended in PBS/0.05% HSA and stored at 4°C in the dark.

Collection of serum samples

A pool of normal human serum (NHS) was obtained by drawing venous blood from six healthy donors (three male and three female donors) into dry glass vials with no additive. The blood samples were left at RT for 2 h, before the serum was collected by centrifugation at 3000*x*g for 15 min, pooled, and stored at -80°C, awaiting further analysis. A pool of umbilical cord serum (UCS) was obtained from umbilical cord blood collected from three individuals. The blood samples were left at RT for 2 h, before the serum was collected by centrifugation at 3000*x*g for 15 min, pooled, and stored at -80°C, awaiting further analysis. Serum samples from a previously described MBL defect patient (46) and a C1q deficient patient (47) were used in the flow cytometry experiments.

Flow cytometry

Relevant controls were included routinely in all experiments, including negative controls samples (without protein/serum), isotype controls (mouse IgG1κ isotype control, mouse IgG2a isotype control, rabbit IgG isotype control, rat IgG1κ isotype control), and background control samples (containing no Ab or only primary or secondary Ab). Samples were analyzed by flow cytometry using a Gallios flow cytometer (Beckman Coulter) and data were analyzed using Kaluza software (Beckman Coulter). CC were gated as an uniform population on the forward site scatter, containing 80-90% of all counts (see supplemental figure).

Binding of MBL to CC

CC (1.0 x 10^6 particles/ml) were incubated with 5% NHS or MBL defect serum or 0.25-4 µg/ml recombinant protein diluted in barbital buffer (5 mM barbital natrium, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing 0.5% heat inactivated fetal calf serum (FCS) (barbital/FCS) for 30 min at 37°C, shaking. The CC were washed in barbital/FCS after each step. Binding of MBL was detected with 0.5 µg/ml (recombinant protein) or 5 µg/ml (serum) mouse anti-human MBL mAb

131-10 (30 min at 4°C), followed by 2.5 µg/ml FITC conjugated polyclonal goat anti-mouse Ab (20 min at 4°C). In some experiments CC were incubated with serum preincubated 5 min at RT with 10 µg/ml MBL inhibitory mAb mouse anti-human MBL 3F8, or 10 µg/ml MBL binding mAb mouse anti-human MBL 1C10, 10 mM EDTA, 10 mM EGTA/Mg, 10 mM GlcNAc, 10 mM D-Mannose, or 10 mM D-(+)-Galactose.

Binding of ficolin-1,ficolin-2, and ficolin-3 to CC

CC (1.0 x 10^6 particles/ml) were incubated with 5% NHS or 0.25-4 µg/ml recombinant protein diluted in barbital/FCS for 30 min at 37°C, shaking. The CC were washed in barbital/FCS after each step. Binding of ficolin-1, -2, or -3 was detected using 5 µg/ml mouse anti-human ficolin-1 FCN106, 0.5 µg/ml (recombinant protein) or 5 µg/ml (serum) mouse anti-human ficolin-2 mAb FCN219, or 5 µg/ml mouse anti-human ficolin-3 mAb FCN334 (30 min at 4°C), followed by 2.5 µg/ml FITC conjugated polyclonal goat anti-mouse Ab (20 min at 4°C). In some experiments CC were incubated with serum preincubated 5 min at RT with 10 µg/ml ficolin-2 inhibitory mAb FCN212, 10 µg/ml acetylated BSA (AcBSA) prepared as described in Hein *et al.* (2010) (48), 10 µg/ml BSA, 10 mM EDTA, 10 mM EGTA/Mg, 20 mM GlcNAc, 20 mM D-Mannose, or 20 mM D-(+)-Galactose.

Binding of MASPs/MAP-1 to CC

CC (1.0 x 10^6 particles/ml) were incubated with 5% NHS diluted in barbital/FCS for 30 min at 37°C, shaking. The CC were washed in barbital/FCS after each step. Binding of MASP-1/-3/MAP-1 was detected using 5 µg/ml mouse anti-human MASP-1/-3/MAP-1 8B3 and MASP-2 binding was detected using 5 µg/ml rat anti-human MASP-2 mAb 8B5 (30 min at 4°C), followed by 2.5 µg/ml

FITC conjugated polyclonal goat anti-mouse Ab or 2 µg/ml FITC conjugated polyclonal rabbit anti-rat Ab (20 min at 4°C).

Binding of MASPs in the presence of ficolin-2 or ficolin-3

CC (1.0 x 10^6 particles/ml) were incubated with recombinant MASP-1 (4 µg/ml), MASP-2 (1 µg/ml), or MASP-3 (1 µg/ml) or recombinant MASP-1, -2, or -3 in the presence of recombinant ficolin-2 (2 µg/ml) or ficolin-3 (2 µg/ml) diluted in barbital/FCS for 30 min at 37°C, shaking. The CC were washed in barbital/FCS after each step. Binding of MASP-1 or MASP-3 was detected using 5 µg/ml mouse anti-human MASP-1/-3/MAP-1 mAb 8B3 (30 min at 4°C), followed by 2.5 µg/ml FITC conjugated polyclonal goat anti-mouse Ab (20 min at 4°C). Binding of MASP-2 was detected using 5 µg/ml rat anti-human MASP-2 mAb 8B5 (30 min at 4°C), followed by 2 µg/ml FITC conjugated polyclonal rabbit anti-rat Ab (20 min at 4°C).

Ficolin-2 mediated C4 deposition

To assess ficolin-2 mediated complement activation CC (1.0 x 10^6 particles/ml) were incubated with recombinant ficolin-2 (2 µg/ml), recombinant MASP-2 (0.5 µg/ml) or a mixture of the proteins (30 min at 37°C, shaking), followed by incubation with purified human C4 (10 µg/ml) (30 min at 37°C, shaking). The CC were washed in barbital/FCS after each step. Deposition of C4b on the CC was detected using 0.5 µg/ml polyclonal rabbit anti-human C4c Ab (30 min at 4°C), followed by 2 µg/ml FITC conjugated polyclonal goat anti-rabbit Ab (20 min at 4°C).

Complement activation on cholesterol crystals, when incubated with serum

Complement activation from serum was assessed by incubating CC (1.0 x 10^6 particles/ml) with 10% NHS, C1q deficient serum with or without reconstitution with 10 µg/ml purified C1q, MBL

defect serum, or UCS diluted in barbital/FCS (30 min at 37°C, shaking). The CC were washed in barbital/FCS after each step. Deposition of C5b-9 was detected using 2 µg/ml mouse anti-human complement component C5b-9 mAb, followed by 2.5 µg/ml FITC conjugated polyclonal goat anti-mouse Ab (20 min at 4°C). In some experiments CC were incubated with serum preincubated 5 min at RT with 50 µg/ml C5 inhibitor (eculizumab), 20 µM C3 inhibitory peptide (compstatin Cp40), 10 µg/ml C1q inhibitory mAb mouse anti-human C1q clone 85, 5 µM MASP-1 inhibitor SGMI-1, 5 µM MASP-2 inhibitor SGMI-2, 10 µg/ml MBL inhibitory mAb mouse anti-human MBL 3F8, or 10 µg/ml MBL binding mAb mouse anti-human MBL 1C10.

Binding of C1q, IgM, and IgG to CC

CC (1.0 x 10^6 particles/ml) were incubated with 5% NHS, C1q deficient serum with or without reconstitution with 10 µg/ml purified C1q, MBL defect serum, or UCS diluted in barbital/FCS for 30 min at 37°C, shaking. The CC were washed in barbital/FCS after each step. Binding of C1q was detected with polyclonal rabbit anti-human C1q (30 min at 4°C), followed by 2 µg/ml FITC conjugated polyclonal goat anti-rabbit Ab (20 min at 4°C). Binding of IgM or IgG was detected using 1 µg/ml rabbit anti-human IgM or 0.1 µg/ml rabbit anti-human IgG, followed by 2 µg/ml FITC conjugated polyclonal goat anti-rabbit Ab (20 min at 4°C). In some experiments CC were incubated with serum preincubated 5 min at RT with 10 µg/ml C1q inhibitory mAb mouse anti-human C1q clone 85.

Microscopy of CC

Microscopy was performed using a Zeiss Axio Observer through a 63x/1.40 oil DIC Plan-Apochromat objective. Imaging conditions were kept constant when acquiring images to be compared. Relevant controls were included routinely in all experiments, including negative controls

samples (without protein), isotype controls (mouse IgG1κ isotype control, mouse IgG2a isotype control), and background control samples (containing no Ab or only primary or secondary Ab).

Binding of recombinant MBL and ficolin-2

CC (2.0 x 10^6 particles/ml) were incubated with recombinant MBL (20 µg/ml) or ficolin-2 (8 µg/ml) diluted in barbital/FCS for 30 min at 37°C, shaking. The CC were washed in barbital/FCS after each step. Binding of MBL and ficolin-2 was detected with 5 µg/ml mouse anti-human MBL mAb 131-10 or 0.5 µg/ml mouse anti-human ficolin-2 mAb FCN219 (30 min at 4°C), followed by 2.5 µg/ml FITC conjugated polyclonal goat anti-mouse Ab (20 min at 4°C). CC were placed on slides by cytospin (centrifugation for 5 min at 300*x*g) and mounted with Glycergel mounting medium.

Human carotid plaques

Carotid plaques used for immunohistochemistry (IHC) and fluorescence microscopy were obtained from patients with carotid stenosis (49). Signed informed consent for participation in the study was obtained from all individuals. Plaque for paraffin sections was immediately after excision put in formalin for fixation before embedded in paraffin. Plaque for frozen sections was taken and immediately put in Tissue-Tek O.C.T. compound and frozen at -80C.

Haematoxylin Erythrosine (H&E) staining

Haematoxylin Erythrosine (H&E) staining was prepared by a histological routine staining protocol. Briefly, formalin fixed, paraffin embedded sections of carotid plaques (4µm) were dried at 60 °C and stained in an automatic slide stainer (Sakura Tissue-Tek © Prisma™, USA). After deparaffinization and rehydration to water the slides were stained with H&E, and rinsed in water for

removal of excess dye. The sections were then dehydrated by adding ascending grades of ethanol and stained in Saffron, rinsed in several baths of absolute ethanol and cleared in Tissue Clear before cover slipping in Sakura Tissue-Tek © Glas™ automatic cover slipper.

Indirect immunofluorescence of carotid sections

Frozen-sections (5 µm) on SuperFrost Plus glass were dried for 10 min and washed in Dako 3006 wash buffer before incubation in Serum Free Protein block for 30 min at RT. Mouse anti-human MBL mAb 131-01 (10 µg/ml), mouse anti-human ficolin-2 mAb FCN219 (8 µg/ml), and corresponding isotype controls were added and incubated over night at 4°C. After washing, the sections were incubated with Alexa647 anti-mouse IgG (3.3 µg/ml) for 60 min at RT before mounting in Mowiol 4-88. The paraffin- and frozen sections were examined in an EVOS FL auto microscope (Thermo Fisher Scientific, USA).

Immunohistochemistry of carotid sections

Dried paraffin embedded sections of carotid plaques (4µm) were deparaffinized and rehydrated by increasing amounts of water in a Sakura Tissue-Tek © Prisma™. The sections were then stained in a Dako EnVision Plus autostainer for mouse antibodies according to the manufacturer protocol. Briefly, endogenous peroxidase activity was blocked by Peroxidase block and incubated with mouse anti-human MBL mAb 131-01 (10 µg/ml), mouse anti-human ficolin-2 mAb FCN219, or an isotype control over night at 4°C followed by Polymer EnVision-HRP anti-mouse for 30 min. A DAB+ Chromogen solution was used to develop the color. The sections were then stained with Hematoxylin, dehydrated and prepared as described for the H&S staining.

312 **Statistical analysis**

313 GraphPad Prism version 6 (Graphpad Software) was used for statistical analysis. Data are expressed
314 as mean ± SEM. Statistical analysis was performed on three independent experiments using two-
315 tailed paired t-test. $P < 0.05$ was considered statistically significant.

316

317 **Ethical approval**

318 The study was approved by the regional health ethics committee in the Capital Region of
319 Denmark (reference no. H2-2011-133). Carotid plaques used for IHC and fluorescence microscopy
320 were obtained from patients with carotid stenosis (49). The protocols were approved by the
321 Regional Committee for Medical and Research Ethics, South-East, Norway (reference no. S-0923a
322 2009/6065).

Results

Binding of lectin pathway pattern recognition molecules

In the first set of experiments we investigated the binding of the PRMs from the LP to CC *in vitro*. No binding of ficolin-1 or ficolin-3 from NHS to CC was observed (Figure 1A, 1C). Ficolin-2 bound strongly to CC, when incubated with NHS (Figure 1B) or recombinant ficolin-2 (Figure 1E and Figure 2). Ficolin-2 binding was inhibited when serum was preincubated with a ficolin-2 inhibitory antibody (Ab) ($p < 0.01$) or specific ligands; AcBSA ($p < 0.01$) or GlcNAc ($p < 0.05$) (Figure 1B). Removal of calcium by addition of EDTA or EGTA/Mg had no inhibitory effect on ficolin-2 binding, but EDTA significantly ($p<0.05$) enhanced ficolin-2 binding. A strong binding of MBL to the CC was observed when incubated with NHS (Figure 1D) or recombinant MBL (Figure 1F and Figure 2). MBL binding was significantly inhibited when serum was preincubated with an MBL inhibitory Ab ($p < 0.01$), specific ligands; GlcNAc ($p < 0.01$) or D-Mannose ($p < 0.05$), or in the absence of calcium (EDTA or EGTA/Mg) ($p < 0.01$) (Figure 1D). No binding of MBL was observed from MBL defect serum. These data show that both ficolin-2 and MBL bind specifically to CC incubated with serum or recombinant proteins.

MASP binding and ficolin-2 mediated C4 deposition

To further investigate the role of the lectin pathway, we looked into the binding of the MASPs and MAP-1 to CC incubated with NHS (Figure 3). Binding of MASP-1/-3/MAP-1 was detected using specific mouse mAb 8B3, recognizing the common heavy chain on all three molecules (41). A significant binding ($p < 0.05$) of native MASP-1/-3/MAP-1 to CC was observed (Figure 3A), whereas no binding of MASP-2 from NHS was observed (Figure 3B). To support this, we determined if the MASPs were able to form complexes with ficolin-2 on the surface of the CC (Figure 4). Recombinant MASPs bound to CC, and the binding of MASP-1 and MASP-3 was

significantly increased ($p < 0.05$) when co-incubated with ficolin-2, but not when co-incubated with the homologue PRM ficolin-3 (Figure 4A). Binding of MASP-2 was also increased when co-incubated with ficolin-2, but the increase did not reach statistical significance (Figure 4A).

We then determined if ficolin-2/MASP-2 complexes were able to activate complement by inducing C4 cleavage. Ficolin-2/MASP-2 complexes significantly increased C4 activation and deposition on the CC compared to CC incubated with C4 alone ($p < 0.05$), ficolin-2 followed by C4 ($p < 0.01$), or MASP-2 followed by C4 ($p < 0.05$) (Figure 4B). MASP-2 alone could be absorbed to the CC without ficolin-2 and enhance the C4 activation and deposition, but the increase did not reach statistical significance. These results indicate that the MASPs bind to CC and form functional complexes with ficolin-2.

Localization of ficolin-2 and MBL in human carotid sections

To confirm the physiological significance of the binding of ficolin-2 and MBL to the CC *in vitro*, we examined the deposition of ficolin-2 and MBL in human carotid plaques by IHC on paraffin embedded sections and immunofluorescence on frozen sections. Ficolin-2 and MBL were deposited in frozen sections of carotid plaques (Figure 5). Furthermore, immunohistochemically staining of MBL in carotid plaques showed colocalization of MBL and CC, visualized as MBL deposition around CC clefts (Figure 6). No staining was observed on any isotype controls. We were not able to detect the presence of ficolin-2 by IHC on paraffin sections, probably because of lack of epitope recognition of the ficolin-2 specific antibody. These results show that ficolin-2 and MBL are present in human carotid plaques and that MBL is localized around CC clefts.

Classical and lectin pathway activation on CC

To further address the involvement of the LP in the CC induced complement activation, we measured the *in vitro* deposition of the complement activation product C5b-9 on CC after incubation with NHS, MBL defect serum, or C1q deficient serum by flow cytometry (Figure 7). In some experiments serum was preincubated with control Ab or different complement inhibitors; C3 inhibitor (compstatin Cp40), C5 inhibitor (eculizumab), C1q inhibitory Ab, MASP-1 inhibitor, MASP-2 inhibitor, MBL control Ab, or MBL inhibitory Ab. C5b-9 deposition on CC was significantly inhibited with the C3 inhibitor compstatin Cp40 ($p < 0.01$) and completely removed by inhibition with the C5 inhibitor eculizumab ($p < 0.01$) (Figure 7A).

C5b-9 deposition on CC from NHS was partly but significantly inhibited ($p < 0.01$) by the C1q inhibitory Ab (Figure 7B + 7C). The remaining complement activation in NHS was inhibited when combining the C1q inhibitory Ab with a LP inhibitor (MBL inhibitory Ab or MASP inhibitor) (Figure 7B and Figure 7C). The MBL inhibitory Ab in combination with the C1q inhibitory Ab significantly reduced ($p < 0.01$) the C5b-9 deposition on CC compared to inhibition with the C1q inhibitory Ab alone or the C1q inhibitory Ab and the MBL binding Ab control (Figure 7B). The MBL inhibitory Ab had no effect on the level of C5b-9 deposition on CC when incubated with NHS without the addition of the C1q inhibitory Ab. The MASP-1 inhibitor in combination with the C1q inhibitory Ab significantly reduced ($p < 0.05$) the level of C5b-9 deposition on CC when incubated with NHS compared to the C1q inhibitory Ab alone (Figure 7C). The MASP-1 inhibitor alone reduced the level of complement activation, but the decrease did not reach statistical significance. The MASP-2 inhibitor alone did not have an effect on the complement activation on CC, but when incubated in combination with the C1q inhibitory Ab, it decreased the level of C5b-9 deposition, but the decrease did not reach statistical significance (Figure 7C). In the MBL defect serum, complement activation on CC was significantly reduced ($p < 0.01$) by preincubating serum with the

C1q inhibitory Ab (Figure 7D). Complement activation on CC from C1q deficient serum was inhibited by the LP inhibitors as in NHS. The MBL inhibitory Ab significantly decreased ($p < 0.05$) the C5b-9 deposition on the CC compared to buffer (Figure 7E). Both the MASP-1 ($p < 0.001$) and the MASP-2 inhibitor decreased the complement deposition on CC from C1q deficient serum (Figure 7F), but the decrease did not reach statistical significance using the MASP-2 inhibitor. C1q reconstitution in the C1q deficient serum significantly increased ($p < 0.05$) the level of complement activation on CC to the level in NHS (Figure 7E). Inhibition of complement activation with the ficolin-2 inhibitory Ab alone or in combination with the C1q inhibitory Ab did not decrease the C5b-9 deposition in NHS (data not shown). Collectively, these data demonstrated that both the CP, initiated by C1q, and the LP, initiated by MBL, play important roles in the initiation of complement activation occurring on CC.

C1q binding to CC is mediated by IgM

It has previously been shown that C1q binds to CC (7). To confirm this in our system and to further study the binding of C1q, we examined the binding of C1q, IgM, and IgG to CC from NHS, C1q deficient, MBL defect serum, and UCS with low amounts of IgM. C1q bound strongly to the CC when incubated with serum (Figure 8). The C1q binding from NHS, MBL defect serum, and UCS was significantly inhibited ($p < 0.05$) with a C1q inhibitory Ab (Figure 8A). No binding of C1q was observed from C1q deficient serum. The C1q binding was reconstituted to the level of NHS when adding purified C1q to the C1q deficient serum. The C1q binding from UCS with low amounts of IgM was significantly lower ($p < 0.01$) than from NHS (Figure 8A). Supporting this, IgM binding to CC was significantly lower ($p < 0.01$) from UCS compared to NHS. IgM binding from C1q deficient serum was significantly increased ($p < 0.05$) compared to NHS. To investigate this, we inhibited C1q binding in NHS and reconstituted C1q in C1q deficient serum. When inhibiting C1q

418 binding in NHS, the detected level of IgM binding increased, whereas reconstitution of C1q in the
419 C1q deficient serum significantly reduced ($p < 0.05$) the detected level of IgM binding to the level
420 of NHS. Inhibition of C1q in MBL defect serum had almost the same effect as in NHS, whereas the
421 level in UCS only slightly increased (Figure 8B). No binding of IgG to CC was observed (Figure
422 8C). These data demonstrate that IgM but not IgG binds to CC when incubated with serum, and
423 indicate that C1q binds indirectly to the CC via IgM.
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425
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Discussion

Crystalized cholesterol is found in early atherosclerotic lesions (1) and the presence of CC is regarded as a hallmark of atherosclerosis (50). CC activate the CP and the AP and induce complement dependent inflammasome activation and cytokine release (1,3-7). In the present study we report novel and important mechanisms by which CC induce complement activation, and to our knowledge, for the first time show that the lectin pathway is involved in the CC induced inflammatory response.

Complement activation is involved in the development of atherosclerotic lesions. Complement activation to the level of C5b-9 has long been known to occur in atherosclerotic plaques (28,31) and several immunoglobulins, complement components and regulators are present in atherosclerotic lesions including IgG, IgM, C1q, and MBL (30,31). In C3- (26,51), C5- (52), C6- (29), and C1q-deficient (32) mice or rabbit models, reduced complement activity affected atherosclerotic development. C6 complement deficiency was shown to protect against development of diet-induced atherosclerosis in rabbits (29), whereas total aortic atherosclerosis, determined by lipid staining, was greater in aortas from C3-deficient mice compared to controls (26). Taken together these results indicate that complement may have proatherogenic or antiatherogenic roles depending on the stage of atherosclerotic development. The role of the CP in atherosclerotic development has been demonstrated in a C1q deficient mouse model of atherosclerosis (32). C1q was found to reduce atherosclerosis development and mediate removal of apoptotic cells. However, deposition of C5b-9 in mouse aortic root lesions was not abolished in the absence of C1q, suggesting involvement of the LP and/or the AP (32).

MBL is present and produced locally by myeloid cells in early experimental atherosclerotic lesions (35). MBL deposition has also been detected in late stage human atherosclerotic lesion, but no local expression of MBL was observed, suggesting a plasma origin MBL in late stage atherosclerosis (35). Our data demonstrate that MBL recognize CC, similar to those found in atherosclerotic lesions, in a specific and calcium dependent manner, leading to full complement activation and deposition of C5b-9 on the CC. This is surprising since MBL is regarded as a typical lectin, recognizing hydroxyl groups on carbohydrates. However, we suggest that MBL binds monohydrate CC via the CRD by recognizing hydroxyl groups exposed on the crystals. The binding of MBL to the CC demonstrated in this study was highly calcium dependent, and was inhibited by a specific inhibitor (an MBL inhibitory Ab) or known MBL ligands (GlcNAc or D-Mannose) strongly suggesting that this is a specific interaction via the CRD domain. No binding of MBL was detected from MBL defect serum, which in fact contains low molecular MBL unable to bind and mediate complement activation via the CRD domain (53). To support these findings we examined the presence of MBL in human carotid plaques. MBL was deposited in carotid plaques and localized around CC clefts. This confirms the physiological significance of the *in vitro* findings and demonstrates a potential role of the MBL mediated LP in the pathophysiology of atherosclerosis. Several population based studies, analyzing either MBL genotypes or resulting serum levels suggest a role for MBL in cardiovascular disease (34,54-60). However the results are contradictory, suggesting either a proatherogenic or antiatherogenic role of MBL in cardiovascular disease. Both MBL and C1q has been found to bind modified low density lipoprotein end enhance clearance by monocytes and macrophages (61,62). In early atherosclerotic lesions MBL may facilitate clearance of endogenous danger signals, such as late apoptotic or necrotic cells, cellular debris, low density lipoprotein, and CC which are associated with atherosclerosis development and plaque stability (20,21,63). In more advanced stages of atherosclerosis, MBL may have a proinflammatory

474 role; hence downregulation of MBL expression in late human lesions may illustrate control of
475 complement activation and inflammation (35).
476
477 Our data also demonstrate that ficolin-2 binds CC independent of calcium and that ficolin-2/MASPs
478 complexes on CC are able to activate C4, leading to C4b deposition on the CC *in vitro*.
479 Furthermore, ficolin-2 was deposited in human carotid plaques. Whether ficolin-2 mainly functions
480 as an opsonin for phagocytosis independent of complement activation or participates in systemic
481 complement activation as C1q and MBL remains to be established. No binding of ficolin-1 or
482 ficolin-3 from serum to the CC was observed in our experiments. Ficolin-1 is mainly found on the
483 surface of monocytes and granulocytes and in secretory granules of neutrophils and monocytes, but
484 has been shown to circulate in serum in very low concentrations (14,40,64). Due to the low level of
485 ficolin-1 in serum, the potential contribution of locally produced ficolin-1 in CC induced
486 inflammation occurring in atherosclerosis remains to be investigated. The ficolins primarily
487 recognize acetylated compounds, such as GlcNAc and acetylated BSA, via their fibrinogen like
488 domain (18,19). Furthermore, ficolin-2 and ficolin-3 have been shown to bind a wide variety of
489 exogenous and endogenous molecular patterns including several strains of encapsulated Gram-
490 positive bacteria (65,66), lipopolysaccharide from Gram-negative bacteria (67), DNA (22),
491 mitochondria (68), and apoptotic cells (23). Ficolin-2 recognizes and binds ligands through four
492 different binding groves with distinct binding properties (S1, S2, S3 and S4), giving ficolin-2
493 unusual recognition versatility. In comparison, ficolin-1 and ficolin-3 only contain one binding
494 grove (S1) (19,69). The more diverse binding capacity of ficolin-2 could explain the calcium
495 independent binding to the CC. Calcium independent interactions of ficolin-2 have also been shown
496 in recognition of pathogens like *Aspergillus fumigatus* and in interactions with endogenous ligands
497 such as pentraxin-3 (70).

The results from the present study demonstrate an additive role of the MBL and the C1q mediated complement activation on CC. In NHS the level of complement activation was inhibited by a C1q inhibitory Ab, but the complement activation was not abolished. The remaining C5b-9 deposition was removed when inhibiting both C1q and MBL mediated complement activation. Furthermore, we found deposition of ficolin-2 and MBL in human carotid plaques and MBL was colocalized to CC clefts. These data suggest that both LP and the CP play an important role in the CC mediated complement activation occurring in atherosclerotic lesions, and that complement inhibition could be an interesting target for treatment of cardiovascular disease. C1q has been shown to bind CC but the binding mechanism has previously been unknown (7). Our data confirm the binding of C1q to CC and further demonstrate that IgM, but not IgG binds to CC, and that the C1q binding is mediated by IgM.

Complement activation on CC was inhibited less effective in our setup with the C3 inhibitor compstatin Cp40 compared to the C5 inhibitor eculizumab. This could partly be explained by the existence of a C3 bypass mechanism resulting in direct cleavage of C5 without activation of C3 as has been proposed by Huber-Lang *et al.* (71-73).

In conclusion our study provides evidence for an important role for the lectin pathway in the inflammatory response induced by cholesterol crystals, and in particular emphasize the role of ficolin-2 and MBL in the cholesterol crystal mediated inflammation occurring during atherosclerotic plaque development.

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764 **Footnotes**

765

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770 [2]Abbreviations used in this article: AP, alternative pathway; AcBSA, acetylated bovine serum
771 albumin; CC, cholesterol crystals; CP, classical pathway; CRD, carbohydrate recognition domain;
772 GlcNAc, N-acetyl-D-glucosamine; IHC, Immunohistochemistry; LP, lectin pathway; MASP(s),
773 MBL/ficolin-associated serine proteases; MBL, mannose-binding lectin; NHS, normal human
774 serum; PRM, pattern recognition molecules; UCS, umbilical cord serum**.**

Figure legends

Figure 1: The PRMs ficolin-2 and MBL bind to CC. Binding of ficolin-1, ficolin-2, ficolin-3, and MBL to CC assessed by flow cytometry. **A+C:** No binding of ficolin-1 and ficolin-3 to CC was observed when incubating with 5% NHS**. B:** Histogram and bar plot showing ficolin-2 binding to CC when incubated with 5% NHS in the presence of buffer, control antibody (10 µg/ml), ficolin-2 inhibitory antibody (10 µg/ml), ligands (10 µg/ml AcBSA, 10 µg/ml BSA, 20 mM GlcNAc, 20 mM D-Mannose, or 20 mM D-(+)-Galactose), EDTA (10 mM) or EGTA/Mg (10 mM). Ficolin-2 binding was inhibited when serum was preincubated with a ficolin-2 inhibitory Ab (fic-2 inhi. Ab) or specific ligands; AcBSA or GlcNAc. EDTA or EGTA/Mg had no inhibitory effect on the ficolin-2 binding, but EDTA significantly ($p<0.05$) enhanced the ficolin-2 binding to CC. **D:** Histogram and bar plot showing MBL binding to CC when incubated with 5% NHS in the presence of buffer, control antibody (10 µg/ml), MBL inhibitory antibody (10 µg/ml), ligands (10 mM GlcNAc, 10 mM D-Mannose, or 10 mM D-(+)-Galactose) , EDTA (10 mM) or EGTA/Mg (10 mM) or 5% MBL defect serum. MBL binding was inhibited when serum was preincubated with an MBL inhibitory Ab (MBL inhi. Ab), specific ligands; GlcNAc and D-Mannose, and in the absence of calcium (EDTA or EGTA/Mg). No MBL binding was observed from MBL defect serum (MBL def. S). **E:** Concentration dependent binding of recombinant ficolin-2 (0.25-4 µg/ml) to CC. **F:** Concentration dependent binding of recombinant MBL (0.25-4 µg/ml) to CC.
Binding was assessed by median fluorescence intensity (MFI) and data are given as mean ± SEM (n=3). Histograms represent one of three independent experiments while columns represent three independent experiments. ns, non-significant, * $p < 0.05$, ** $p < 0.01$ compared to buffer or otherwise indicated.

Figure 2: Binding of recombinant ficolin-2 and MBL to CC. Binding of ficolin-2 (8 µg/ml) and MBL (20 µg/ml) to CC assessed by fluorescence microscopy. Imaging conditions were kept constant when acquiring images to be compared. Results are representative of three independent experiments.

Figure 3: MASP-1/-3/ MAP-1 binding from serum. Binding of MASP-1/-3/MAP-1 or MASP-2 to CC assessed by flow cytometry. **A:** Histogram and barplot showing significant MASP-1/-3/MAP-1 binding to CC when incubated with 5% NHS. **B:** No binding of MASP-2 was observed when CC was incubated with 5% NHS. Binding was assessed by median fluorescence intensity (MFI) and data are given as mean ± SEM (n=3). Histograms represent one of three independent experiments while columns represent three independent experiments. ns, non-significant, * $p < 0.05$ compared to control.

Figure 4: Ficolin-2 mediates MASP binding and C4 deposition on CC. A: Binding of recombinant MASP-1 (4 µg/ml), MASP-2 (1 µg/ml), or MASP-3 (1 µg/ml) to CC assessed by flow cytometry. Binding of MASP-1, MASP-2, and MASP-3 was increased in the presence of recombinant ficolin-2 (2 µg/ml) but not in the presence of the homologue PRM ficolin-3 (2µg/ml). **B:** C4 activation and deposition induced by ficolin-2/MASP-2 complexes. CC were incubated with recombinant ficolin-2 (2 µg/ml) and/or recombinant MASP-2 (0.5 µg/ml), followed by incubation with purified human C 4 (10 µg/ml). MASP binding and C4 deposition was assessed by median fluorescence intensity (MFI) and data are given as mean ± SEM (n=3). Results are representative of three independent experiments. ns, non-significant, * $p < 0.05$, ** $p < 0.01$ compared to C4 (Figure 4B) or otherwise indicated.

Figure 5: Ficolin-2 and MBL are present in human carotid plaques. Frozen sections of a human carotid plaque stained with H&E for histological examination (A+D), a ficolin-2 mAb (B+E), or an anti-MBL mAb (C+F). Representative sections of two positions in a carotid plaque are shown. Scale bars represent 200 µm.

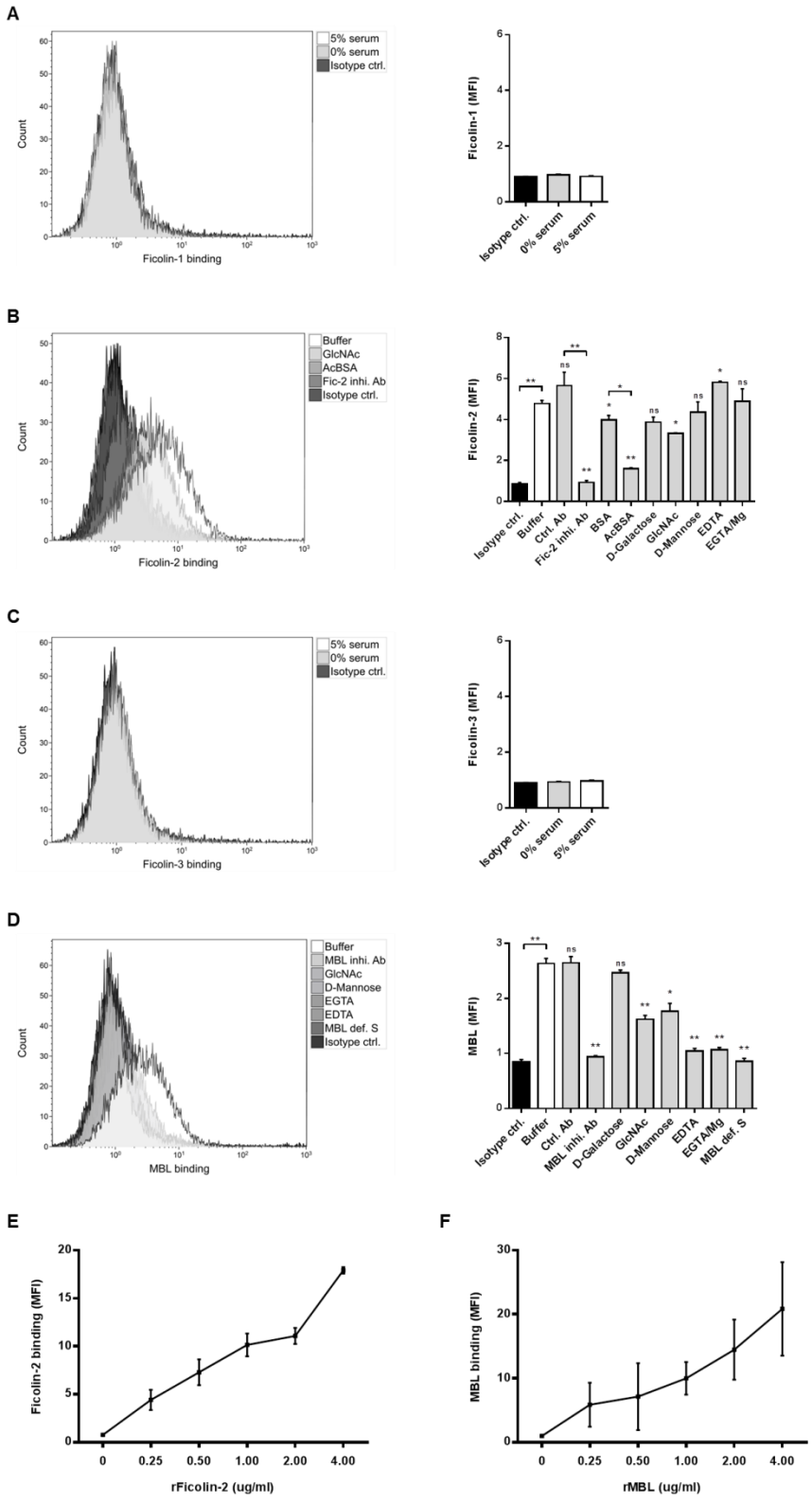
Figure 6: Colocalization of MBL and CC in human carotid plaques. Section of human carotid plaque immunohistochemically stained with an anti-MBL mAb (A+B) or an isotype control (C). Sections were counterstained with hematoxylin**. A+B:** MBL was present in human carotid plaques and localized around CC clefts. **C:** No staining was observed on the isotype control. Scale bars represent 200 µm (A) or 100 µm (B+C).

Figure 7: CC activate the classical and the lectin complement pathway. Complement activation measured by C5b-9 deposition on CC, assessed by flow cytometry. 10% NHS (A-C), MBL defect serum (D) or C1q deficient serum (E-F) was incubated with CC in the presence of buffer, control antibody (10 µg/ml) or different complement inhibitors; C3 inhibitor (20 µM), C5 inhibitor (50 µg/ml), C1q inhibitory Ab (C1q inhi. Ab) (10 µg/ml), MASP-1 inhibitor (MASP-1 inhi.) (5 µM), MASP-2 inhibitor (MASP-2 inhi.) (5 µM), MBL ctrl. Ab (10 µg/ml), or MBL inhibitory Ab (MBL inhi. Ab) (10 µg/ml). **A:** Complement activation on CC was significantly inhibited with the C3 inhibitor (compstatin Cp40) and completely removed by inhibition with the C5 inhibitor (eculizumab). **B+C:** Complement activation in NHS was partly inhibited by the C1q inhi. Ab. The remaining complement activation in NHS was inhibited when combining the C1q inhi. Ab with a LP inhibitor (MBL inhi. Ab (B) or MASP inhi. (C)). **D:** Complement activation in MBL defect serum was inhibited by the C1q inhi. Ab. **E+F:** In the C1q deficient serum, complement activation was inhibited by the LP inhibitors, as in NHS. C5b-9 deposition was assessed by median

fluorescence intensity (MFI) and data are given as mean ± SEM (n=3). Results are representative of three independent experiments. ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8**: C1q binding to CC is mediated by IgM.** C1q, IgM, and IgG binding to CC, when incubated with 5% NHS, C1q deficient serum (C1q def. S), MBL defect serum (MBL def. S), or UCS, measured by flow cytometry. **A:** Strong binding of C1q was observed when incubating CC with 5% NHS. C1q binding from C1q def. S, MBL def. S, and UCS was significantly lower than in NHS. No binding of C1q was observed from C1q def. S. The C1q binding was reconstituted to the level of NHS when adding purified C1q (10 µg/ml) to the C1q def. S. Binding of C1q from NHS, MBL def. S, and UCS was completely inhibited with a C1q inhibitory Ab (C1q inhi. Ab) (10 µg/ml). **B:** Detection of IgM binding to CC when incubated with serum was significantly increased in C1q def. S compared to NHS. When inhibiting C1q binding in NHS the detected level of IgM binding increased. Reconstitution of C1q in the C1q def. S significantly reduced the detected level of IgM binding to the level of NHS. Inhibition of C1q in MBL def. S had the same effect as in NHS, whereas the level in UCS only slightly increased. **C:** No binding of IgG to CC was observed. Binding was assessed by median fluorescence intensity (MFI) and data are given as mean ± SEM (n=3). Results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to NHS or otherwise indicated.

Figure 1


A
5% serum
0% serum
Isotype ctrl.
Count
Ficolin-1 binding
Ficolin-1 (MFI)
Isotype ctrl.
0% serum
5% serum
B
Buffer
GlcNAc
AcBSA
Fic-2 inhi. Ab
Isotype ctrl.
Count
Ficolin-2 binding
Ficolin-2 (MFI)
Isotype ctrl.
Buffer
Ctrl. Ab
Fic-2 inhi. Ab
BSA
AcBSA
D-Galactose
GlcNAc
D-Mannose
EDTA
EGTA/Mg
C
5% serum
0% serum
Isotype ctrl.
Count
Ficolin-3 binding
Ficolin-3 (MFI)
Isotype ctrl.
0% serum
5% serum
D
Buffer
MBL inhi. Ab
GlcNAc
D-Mannose
EGTA
EDTA
MBL def. S
Isotype ctrl.
Count
MBL binding
MBL (MFI)
Isotype ctrl.
Buffer
Ctrl. Ab
MBL inhi. Ab
D-Galactose
GlcNAc
D-Mannose
EDTA
EGTA/Mg
MBL def. S
E
Ficolin-2 binding (MFI)
rFicolin-2 (ug/ml)
0
0.25
0.50
1.00
2.00
4.00
F
MBL binding (MFI)
rMBL (ug/ml)
0
0.25
0.50
1.00
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4.00

Figure 2

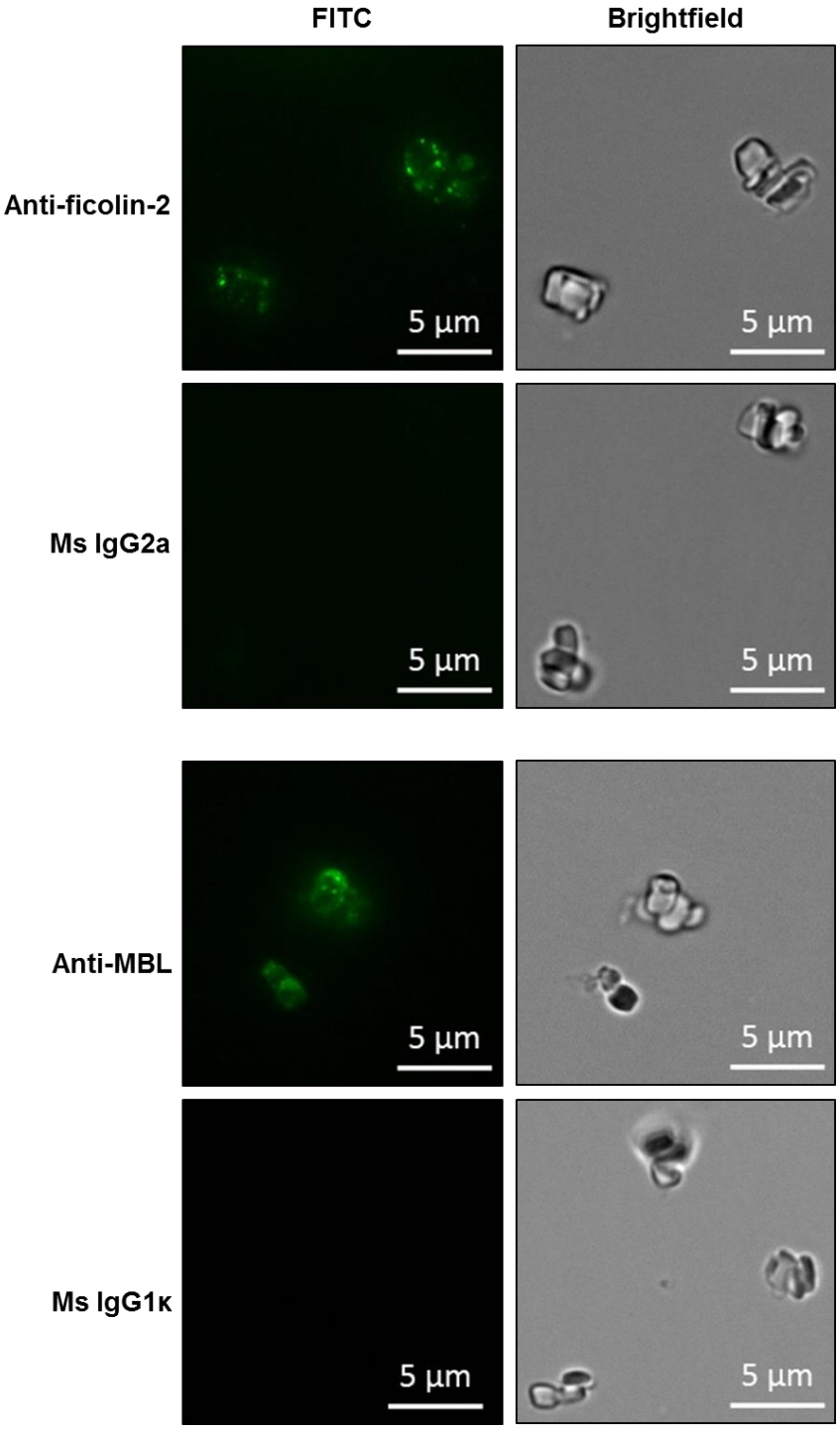

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Figure 3

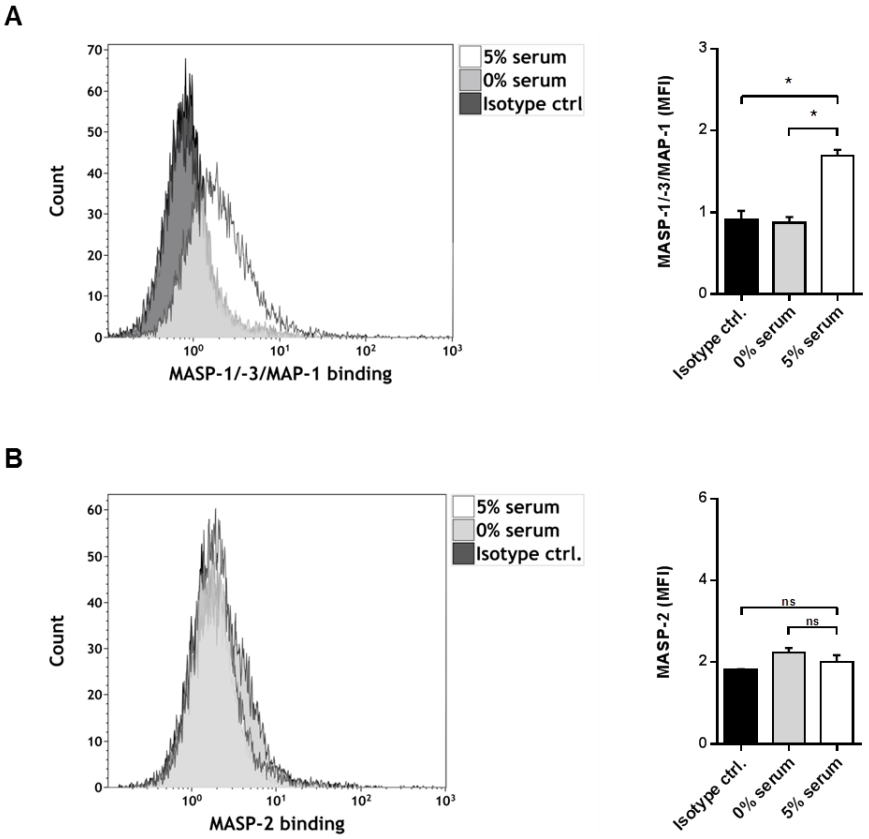

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Figure 4

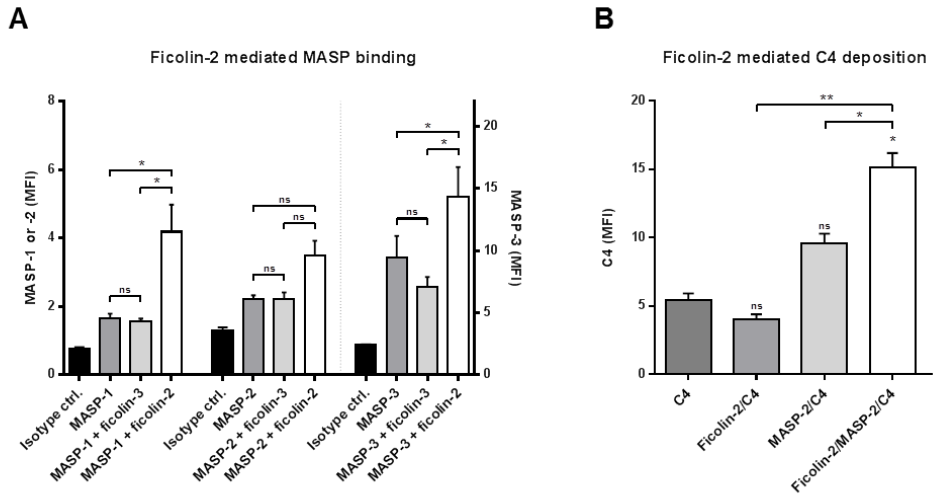

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Figure 5

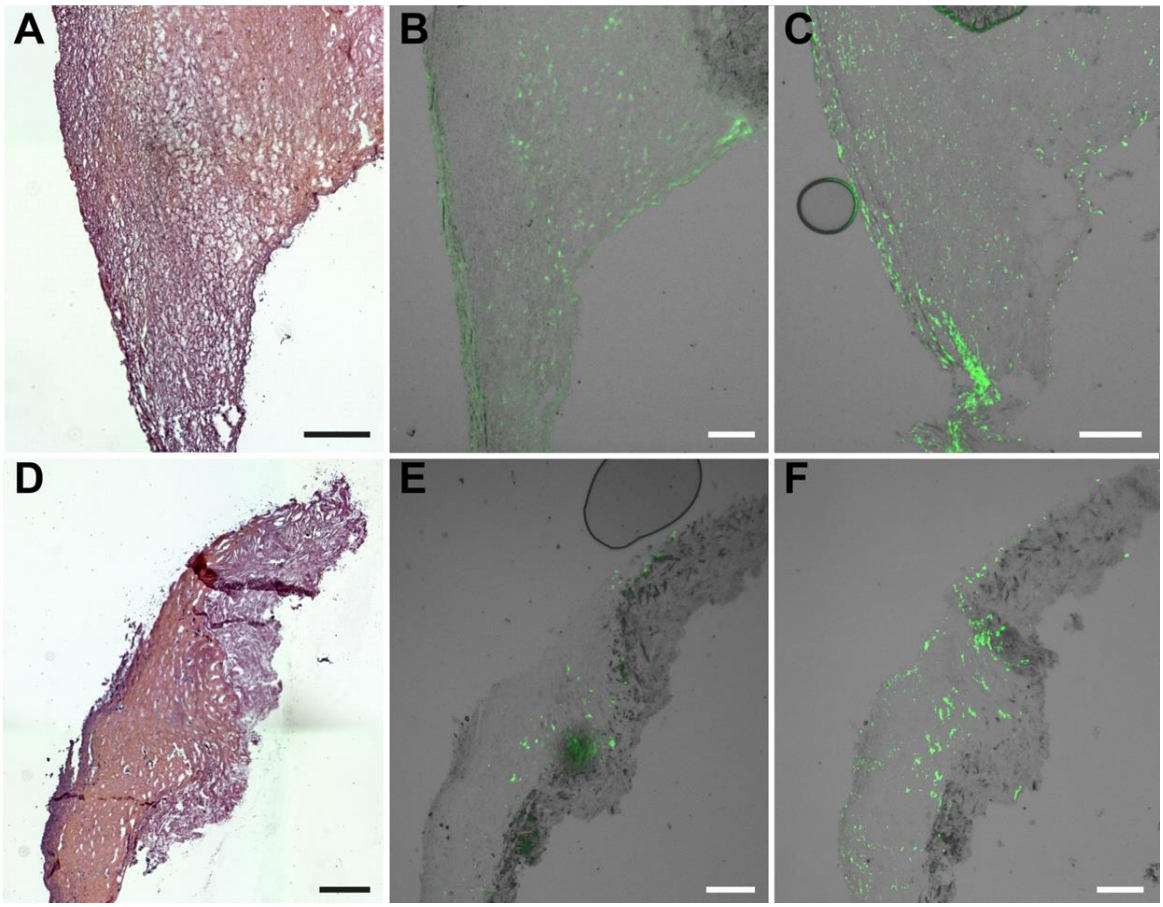

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Figure 6

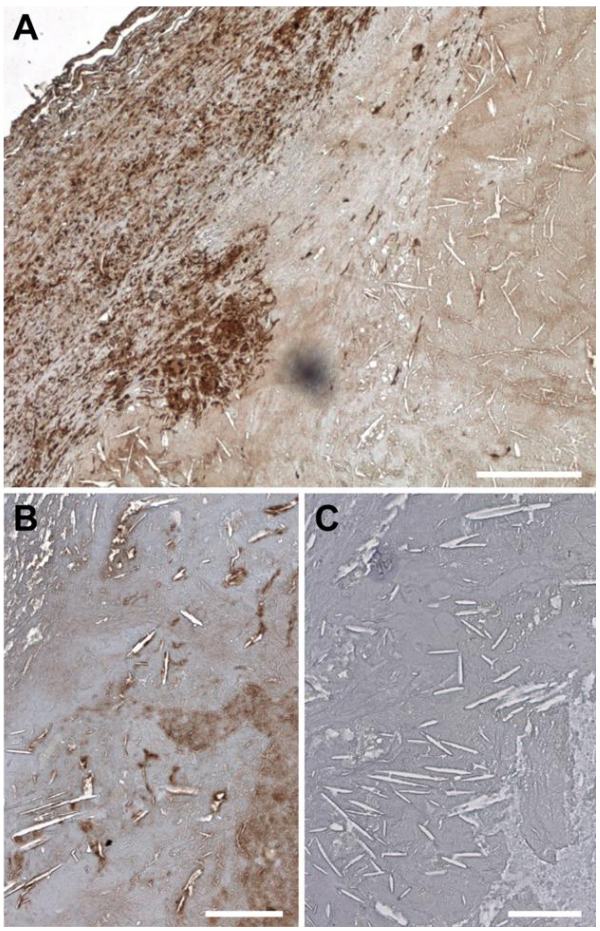

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Figure 7

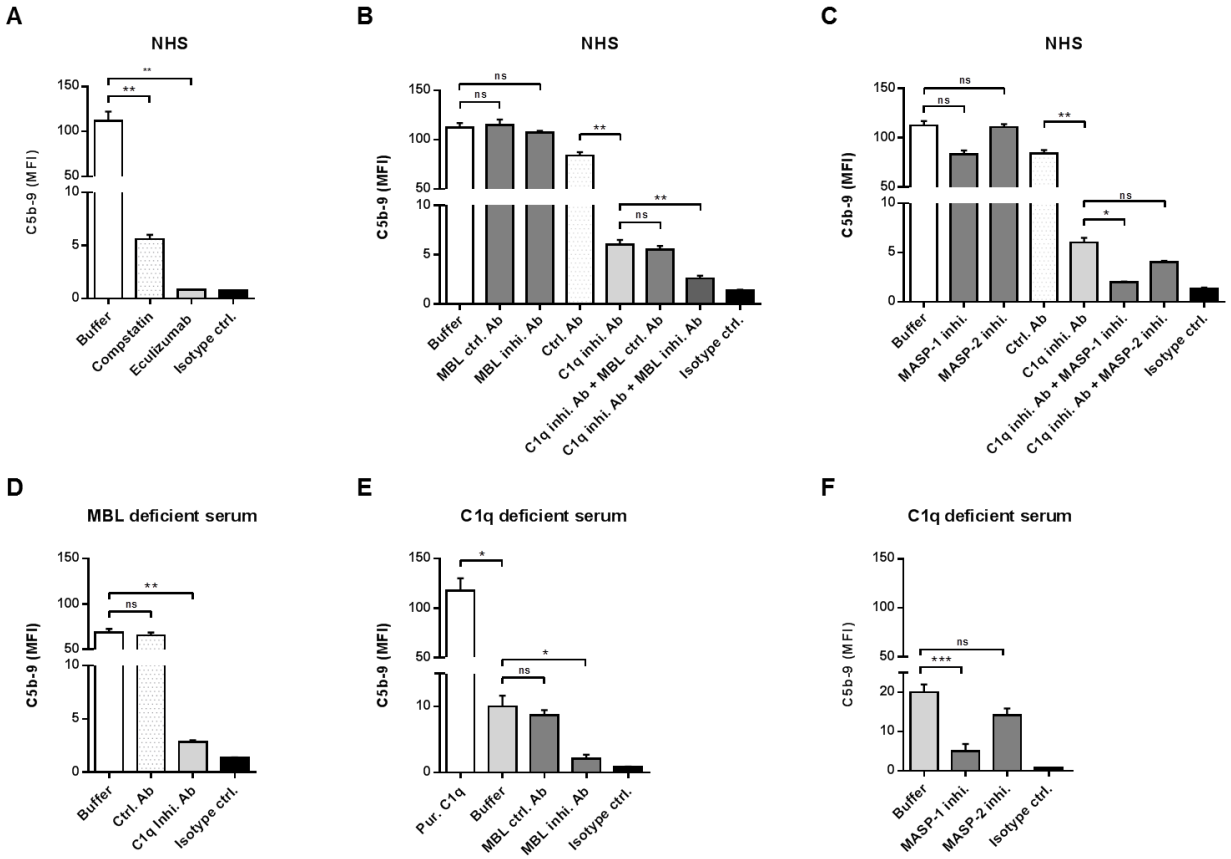

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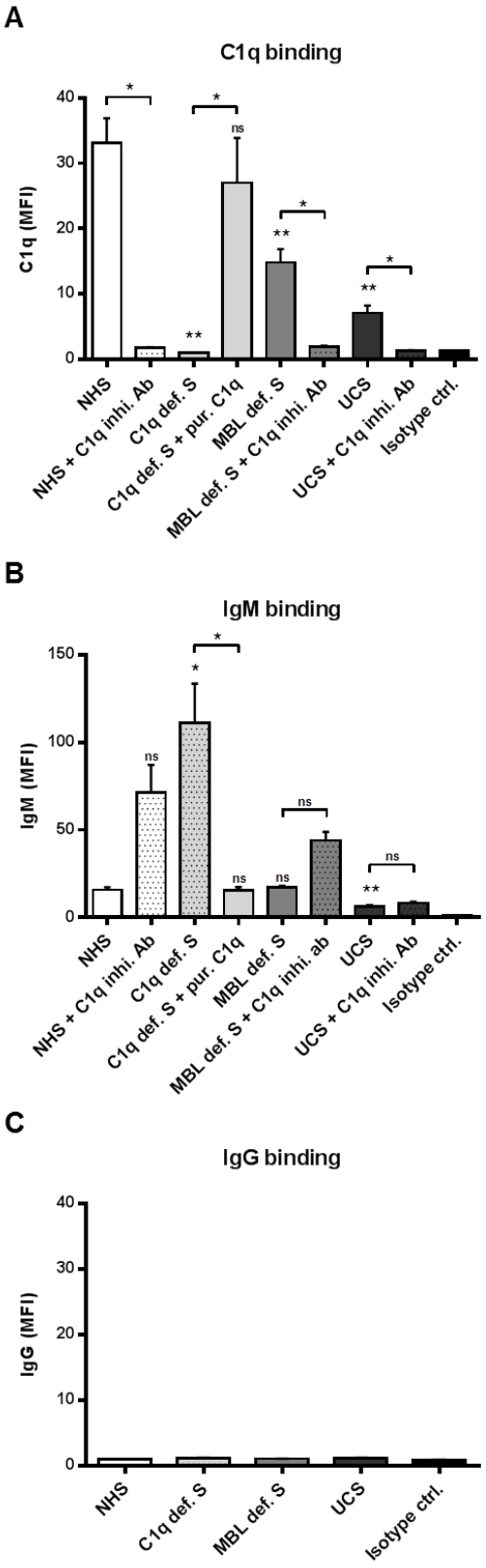
Figure 8


A
C1q binding
C1q (MFI)
40
30
20
10
0
*
*
ns
**
*
**
*
NHS
NHS + C1q inhi. Ab
C1q def. S
C1q def. S + pur. C1q
MBL def. S
MBL def. S + C1q inhi. Ab
UCS
UCS + C1q inhi. Ab
Isotype ctrl.
B
IgM binding
IgM (MFI)
150
100
50
0
*
*
ns
ns
ns
ns
**
ns
NHS
NHS + C1q inhi. Ab
C1q def. S
C1q def. S + pur. C1q
MBL def. S
MBL def. S + C1q inhi. ab
UCS
UCS + C1q inhi. Ab
Isotype ctrl.
C
IgG binding
IgG (MFI)
40
30
20
10
0
NHS
C1q def. S
MBL def. S
UCS
Isotype ctrl.

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Supplemental figure